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Characterization of an isolated chloroplast membrane Fe-S protein and its identification as the photosystem I Fe-S_A/Fe-S_B binding protein

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An iron-sulfur protein of approx. 9 kDa has been isolated from spinach chloroplast membranes. Based on iron and sulfide content (~900 nmol Fe and S^{2-} per mg protein), optical absorbance and low-temperature EPR spectra, it appears that this protein contains 8Fe and $8S^{2-}$ in two [4Fe-4S] clusters. The protein cross-reacts with an antibody raised against a 9 kDa PS I subunit previously identified as the *psaC* (*frxA*) gene product. This identity was confirmed by the N-terminal amino acid sequence of the isolated Fe-S protein. It is concluded that the isolated Fe-S protein binds PS I centers Fe-S_A and Fe-S_B and that each of these centers contains 4Fe and $4S^{2-}$.

Iron-sulfur center; Photosystem I, Ferredoxin; P700

1. INTRODUCTION

Photosystem I (PS I) is known to contain several bound iron-sulfur centers whose functions are to accept electrons released during the photooxidation of the reaction center chlorophyll, P700 [1,2]. Three iron-sulfur centers, denoted Fe-S_X, Fe-S_A, Fe-S_B, have been identified on the basis of their characteristic low-temperature EPR spectra [1,2]. The identity of the proteins binding these centers in PS I is currently an area of active investigation in several laboratories. Golbeck and co-workers [3–5] as well as Hoj and Moller [6] have recently provided evidence that Fe-S_X is associated with the P700-chlorophyll *a*-proteins of the PS I complex. These high-molecular-mass subunits of ~80 kDa are known to contain several other early electron acceptors (A_0 and A_1) as well as P700 [7–9]. It has been proposed by Hoj et al. [10] that a 9 kDa PS I subunit is the apoprotein of Fe-S_A and Fe-S_B. This suggestion was originally put forward by Lagoutte et al. [11] and also more recently by Oh-oka et al. [12] and Hayashida et al. [13]. In these studies, one argument for this assignment was the amino acid sequence of the 9 kDa subunit. The 9 kDa subunit, the gene product of the chloroplast *frxA* or *psaC* gene, has an arrangement of cysteine residues that is strikingly similar to that found in bacterial ferredoxins which contain 2[4Fe-4S] clusters [10,12,13]. The two clusters of cysteines in this protein are presumed to bind Fe-S_A and Fe-S_B. However, this protein has not yet been isolated with intact Fe-S clusters so this assignment remains tentative.

In the present report, a membrane-bound Fe-S protein has been isolated and characterized. In fact, this protein is identical to one previously isolated in this laboratory [14]. Although no function could be assigned at the earlier time, the present results, using specific antibodies and amino acid sequencing, show that the protein, which contains approx. 8Fe and $8S^{2-}$, is the 9 kDa subunit of PS I characterized previously in its apoprotein state by Hoj et al. [10] and Oh-oka et al. [12].

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2. MATERIALS AND METHODS

2.1. *Isolation of a chloroplast membrane Fe-S protein*

The preparation of chloroplast membranes from spinach and the extraction of the lyophilized membranes with methanol plus acetone was as previously described [14]. The dried residue from the solvent extraction was resuspended in ~200 ml of 50 mM Tris-HCl (pH 7.8) + 0.1% β-mercaptoethanol (TM buffer). This suspension was stirred at 4°C for 1 h and then centrifuged at 40000 × *g* for 15 min to obtain the solubilized protein fraction. The extract was applied to a 3 × 15 cm DEAE-Biogel column equilibrated with TM buffer. The column was developed with a linear NaCl gradient in TM buffer (0–0.15 M NaCl, 200 ml in each chamber). Fractions were assayed for S^{2-} and the major peak concentrated with an Amicon YM-5 membrane. For further purification, the pooled DEAE-Biogel fraction was applied to a 2.5 × 100 cm Sephadex G-50 column equilibrated with TM buffer. Sulfide-containing fractions were concentrated as before. Aliquots were stored at −20°C.

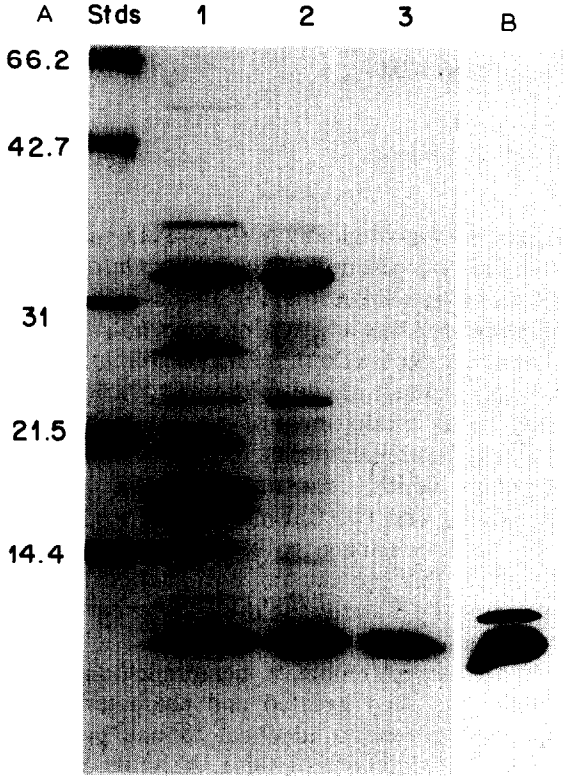

Fig.1. SDS-PAGE and immunoblot analysis of the purified chloroplast membrane Fe-S protein. (A) SDS-PAGE analysis of Fe-S protein fractions. Lanes: 1, extract; 2, DEAE-Biogel fraction; 3, G-50 fraction. Approx. 10 μg protein was loaded in each lane and the gel was stained with Coomassie blue. (B) Immunoblot of the purified G-50 Fe-S protein with an antibody against the PS I *psaC* gene product.

2.2. *Spectroscopy*

Absorption spectra were recorded with a Cary model 219 spectrophotometer at 25°C. EPR spectra were recorded at liquid helium temperatures with a Bruker X-band spectrometer, as described in [15].

2.3. *Analytical methods*

Non-heme iron and acid-labile sulfide determinations were done as described [14]. Protein was based on the absorbance at 205 nm using an absorbance of 31 for a concentration of 1 mg/ml [16] or by the BCA colorimetric procedure [17]. Amino acid sequence analysis was performed using a vapor-phase microprotein sequencer (Applied Biosystems, model 470A) with an on-line, microbore PTH-amino acid analyzer (Applied Biosystems, model 120-A).

2.4. *Electrophoresis and immunoblotting*

SDS-PAGE was done by the method of Laemmli [18] using a 10–20% resolving gradient gel. Immunoblotting was carried out essentially as in [19] utilizing an antibody prepared to the *psaC* gene product of PS I (~9 kDa), kindly supplied by Professor H. Matsubara of Osaka University.

3. RESULTS

3.1. *Purification and properties of the bound Fe-S protein*

SDS-PAGE analysis of the isolated Fe-S protein at various stages of purification are shown in fig.1A. After ion-exchange chromatography on DEAE-Biogel, a low-molecular-mass protein was the major component with some higher molecular mass contaminants also present. The latter could be removed by gel filtration to yield a preparation

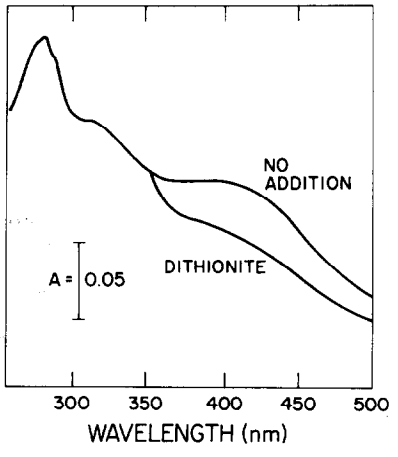

Fig.2. Absorbance spectra of the purified G-50 Fe-S protein. Spectra were recorded at 25°C at a protein concentration of ~0.1 mg/ml using a 2 mm light path. The protein was reduced by the addition of a small amount of sodium dithionite.

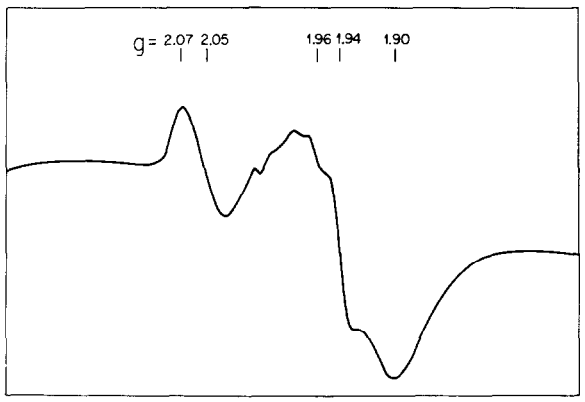

Fig.3. EPR spectrum of the purified G-50 Fe-S protein. The spectrum was recorded at 10K using the following instrument conditions: field, 3450 ± 500 G; power, 10 mW; modulation amplitude, 10 G. The protein concentration was ~0.5 mg/ml and the sample was reduced with a small amount of sodium dithionite prior to freezing.

which was highly purified. Under the SDS-PAGE conditions used, the protein showed a molecular mass of approx. 9 kDa. In a non-denaturing gel, a single brown band was observed during electrophoresis of the purified protein. The native protein was relatively instable as evidenced by its loss of visible absorbance during the purification and during storage at 4°C. For this reason, the purification procedure was done as quickly as possible after the protein had been solubilized from the membranes, and aliquots of purified protein stored at −20°C.

The absorbance spectrum of the native protein is shown in fig.2. This spectrum shows a broad band at ~400 nm, a shoulder at ~320 nm and several distinctive shoulders in the ultraviolet region (290 and 280 nm) in addition to a protein peak at 282 nm. The ratio of absorbance at 400:282 was 0.59 for the purified prepafation. The addition of sodium dithionite caused a bleaching of the visible absorbance bands and this could be reversed by mixing the sample with air. The EPR spectrum of the dithionite-reduced protein is shown in fig.3. The oxidized sample showed no EPR signals while the reduced sample showed a complex signal with apparent transitions at g = 2.07, 2.05, 1.96, 1.94 and 1.90.

Analysis of the purified protein for non-heme iron gave values from 800 to 1000 nmol Fe per mg protein. Values are for three different preparations of the protein. Sulfide contents ranged from 600 to 900 nmol sulfide per mg protein. Assuming a molecular mass of 8.9 kDa for the purified protein leads to average values of 7.9 mol Fe and 6.4 mol sulfide per mol protein. The lower value for sulfide content may reflect the presence of some apoprotein which has lost sulfide due to the instability of the iron-sulfur centers while the excess iron is retained in a non-specifically bound state.

3.2. *Association of the purified Fe-S protein with the* psaC *gene product of PS I*

The results shown in fig.1B indicate that purified Fe-S cross-reacts with an antibody raised against the *psaC* gene product of PS I. Oh-oka et al. [12] isolated this subunit from a spinach PS I preparation and prepared an antibody against the denatured protein. A cross-reacting product of ~9 kDa was also detected in the pooled DEAE-Biogel fraction and in the crude extract. A minor

	AMINO ACID RESIDUE														
	1				5				10					15	
Deduced Sequence-Liverwort psaC gene product	A	H	A	V	K	I	Y	D	T	C	I	G	C	T	Q	C	V
Deduced sequence-tobacco psaC gene product	S	H	S	V	K	I	Y	D	T	C	I	G	C	T	Q	C	V
Spinach ~8 kDa PSI subunit	S	H	S	V	K	I	Y	D	T	C	I	G	C	T	Q	C	V
Barley ~9 kDa PSI subunit	S	H	S	V	K	I	Y	D	T	C	I	G	C	T	Q	C	V
Isolated chloroplast membrane Fe-S protein	S	-	S	V	K	I	Y	D	T	-	I	G	-	T	Q		

Fig.4. N-terminal amino acid sequence of the purified Fe-S protein and its comparison with the sequence of a 9 kDa PS I subunit from spinach [12] and barley [10] and the *psaC* gene products from tobacco [13] and liverwort [20].

cross-reacting product of approx. 10 kDa was detected in the purified fraction even though this band is barely detected in the stained gel (fig.1A). The nature of this cross-reacting contaminant is unclear. The immunoblotting results indicate that the 9 kDa Fe-S protein cross-reacts with the *psaC* gene product isolated from PS I by Oh-oka et al. [12], and these results identify the purified Fe-S protein as the *psaC* gene product.

A further confirmation that the purified protein is the *psaC* gene comes from a comparison of its N-terminal amino acid sequence with published sequences. As summarized in fig.4, the isolated Fe-S protein has an identical sequence to the apoprotein isolated by Oh-oka et al. [12] from spinach PS I and the corresponding PS I subunit isolated from barley PS I by Hoj et al. [10]. The sequences of the *psaC* gene products from tobacco and liverwort are also included in this figure. Unfortunately the sequencing method used in the present work did not allow determination of cysteine or histidine, but even with these omissions, it is clear that these proteins in barley and spinach, or gene products in the case of tobacco and liverwort, are identical.

4. DISCUSSION

The results of the present work identify an isolated Fe-S protein as the *psaC* gene product previously characterized by two groups in its iron and sulfide-free apoprotein form [10,12]. The visible absorbance spectrum of the protein is similar to isolated Fe-S proteins that contain at least 4 mol of non-heme iron and four mol of sulfides [21] while the EPR signal, showing more than 3 *g* values, is reminiscent of bacterial type ferredoxins which contain 2[4Fe-4S] clusters [22–24]. Analytical results on iron and sulfide content also indicate that the isolated protein probably contains 2[4Fe-4S] clusters but the instability of the centers and the uncertainty in determination of the protein content based on colorimetric method makes this conclusion more tentative.

The remaining question concerning this isolated Fe-S protein is its presumed identity as the Fe-S_A/Fe-S_B binding protein. The EPR *g* values of the isolated protein are not the same as those for Fe-S_A and Fe-S_B in situ but this may reflect alteration in the Fe-S clusters after removal from the membrane. Thus, the EPR data do not allow for a conclusive association with Fe-S_A and Fe-S_B. Hoj et al. [10] have shown that only two PS I subunits bind zero-valence sulfur, the denatured form of sulfide. One subunit is the high-molecular-mass P700-chlorophyll-protein while the second is the 9 kDa PS I subunit. Since PS I contains no additional sulfide-containing subunits, it is likely both Fe-S_A and Fe-S_B are coordinated to the same subunit to yield an 8Fe-8S protein. The finding that the isolated Fe-S protein contains approx. 8Fe and $8S^{2-}$ and is the *psaC* gene product strongly supports our contention that this protein binds Fe-S_A and Fe-S_B in two [4Fe-4S] clusters.

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